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Selective Culture Medium to Survey the Incidence of *Haemophilus* Species[1]

JAMES J. CRAWFORD, LOUISE BARDEN, AND JAMES B. KIRKMAN, JR.[2]

Department of Endodontics, Dental Research Center, University of North Carolina School of Dentistry, Chapel Hill, North Carolina 27514

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A culture medium for the selective isolation of *Haemophilus* species is described. Bacitracin and nutritional supplements were incorporated in a rich basal agar medium to which rabbit blood was added to distinguish hemolytic species. Colony counts of seven typed strains of *H. influenzae* on this medium were within practical limits of counts on other media tested for clinical use. The bacitracin medium was as reliable as hemoglobin-agar for detecting *H. influenzae* and more sensitive for detecting other *Haemophilus* species in a clinical survey with the advantage of selectivity.

Need for a selective medium to assist in detecting growth of *Haemophilus* species became apparent when we undertook a survey of the carrier incidence of pathogens in the upper respiratory passages of selected groups of children. These were children with palatal clefts who exhibited a high incidence of chronic upper respiratory infections and control subjects. A selective medium was needed to aid the rapid detection and differentiation of hemolytic and nonhemolytic *Haemophilus* colonies in the heavy mixed growth obtained from oropharyngeal and nasopharyngeal samples.

This report describes a nutritionally supplemented basic medium formulation enriched with fresh rabbit blood and made selective by the addition of antibiotics, and its comparison with other formulations for the cultivation of stock *H. influenzae* strains and the isolation of new strains from clinical specimens.

MATERIALS AND METHODS

Microorganisms. Typed strains of *H. influenzae* were obtained from the American Type Culture Collection. ATCC 9334, 9795, and 10211 were listed as type b strains; ATCC 8142 as type e; and ATCC 9833 as type f. All originated from the laboratory of Margaret Pittmen at the National Institutes of Health. These strains and two untyped clinical isolates that required both hematin and diphosphopyridine nucleotide (X and V factors) were used in initial quantitative medium comparison tests.

Other strains were those that occurred in clinical oropharyngeal specimens obtained from the patients examined.

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[2] Present address: Division of Infectious Diseases, Department of Medicine, University of North Carolina School of Medicine, Chapel Hill, N.C. 27514.

Nutritional characterization of strains. Nutritional growth requirements for X and V factors were determined by use of commercial X and V factor test discs (Difco). Determinations were confirmed by growth tests on Casman agar (Difco) prepared with yeast extract, with hemoglobin, and with both yeast extract and hemoglobin.

Media. Six culture medium formulations were utilized: (i) "Basic medium formulation" contained Casman medium sterilized and cooled to 45 C, plus 1 ml of Supplement C (Difco) and 5% (v/v) fresh rabbit blood (Robbin Laboratory, Chapel Hill, N.C.; reference 7). (ii) "Selective medium A" contained the same formulation as the basic medium plus 5 units of bacitracin (Pfizer) per ml. (iii) "Selective medium B" contained the same formulation as the basic medium plus 0.3 units of penicillin (Pfizer) per ml. (iv) "Hemoglobin-agar" contained GC Medium Base (Difco) plus 1% hemoglobin and 1% Supplement C used in broad surveys by Glezen et al. (6) and Loda et al. (8). (v) "Clear medium", essentially a modified Levinthal's medium, was prepared from Casman medium plus 1% Supplement C. This was utilized to detect iridescent colonies produced by encapsulated *Haemophilus* strains on a clear medium. "Plain rabbit blood-agar" was prepared by adding 5% fresh rabbit blood to sterilized Trypticase Soy Agar (BBL) cooled to 45 C. (2). This medium is used in some hospital laboratories for detection of *Haemophilus* species in respiratory specimens.

Quantitative media comparison procedure. The typed ATCC specimens and the two smooth colony laboratory isolates were collected from the surface of plain rabbit blood-agar and suspended in Trypticase Soy Broth. Serial 10-fold dilutions were prepared in the Trypticase Soy Broth. From each dilution, 20-μliter drops were dispensed on three plates of each test medium, with a micropipetting device (Eppendorf Micropipette, Brinkman Instruments, Westbury, N.Y.) by the procedure evaluated by Miles and Misra (9). It was essential that plates be "dried"

prior to use according to their recommendations to prevent colonies from running together. Plates were incubated for 24 hr at 37 C with 5% carbon dioxide. Colony counts were made from the dilution spotted on each plate that produced 20 to 150 colonies per spot. Colonies were counted with the aid of a dissecting microscope at a magnification of 20.

Clinical samples. Clinical respiratory specimens used for media comparisons were obtained from patients with cotton swabs and placed directly into 2 ml of Trypticase Soy Broth containing 1% (v/v) fresh frozen rabbit serum. The tubes were agitated at 20,000 vibrations per sec at 3 amp for 30 sec with the sterile microtip of a 75-w oscillator (Branson Instruments, Inc., Stanford, Conn.). This was predetermined to yield the highest counts of organisms from clinical specimens without producing significant loss of organisms due to rupture. Two selective medium formulations were compared with the basal medium and hemoglobin-agar in their ability to support growth from suspensions of the *H. influenzae* strains.

RESULTS

The *H. influenzae* ATCC stock strains harvested from plain rabbit blood-agar and diluted in Trypticase Soy Broth produced generally uniform, smooth colonies, 1 to 2 mm in diameter, on the supplemented basic medium and on the hemoglobin-agar (Fig. 1). The same suspensions usually produced only pinpoint rough colonies, 0.2 to 0.5 mm in diameter, when inoculated on the nonsupplemented plain rabbit agar medium. On the selective medium A (containing bacitracin), colonies resembled those on the basic medium (Fig. 1). On selective medium B (containing penicillin), colony size was erratic, varying between pinpoint rough colonies seen on the plain rabbit blood-agar to the large size observed on the supplemented basic medium. Means of colony counts and standard deviations obtained on three plates of each medium are recorded for each of seven *H. influenzae* test strains (Fig. 2). Despite uniformity of colony characteristics on medium A, two strains (laboratory isolate II and the ATCC 9334, type b) produced significantly lower counts on medium A than on the other media. Although the numbers of colonies that developed on medium A were significantly lower for two strains, these variations were less than one-half of a log unit. This was judged to be within acceptable limits for qualitative clinical use and to permit the use of medium A to detect differences of one log unit or more among *Haemophilus* populations that could be cultivated from clinical survey specimens.

Qualitative recovery of *Haemophilus* species from clinical specimens on selective medium A was compared with their recovery on hemoglobin-agar and a clear, supplemented basal medium. The latter medium was included to utilize the irridescent character of smooth colonies produced by encapsulated *Haemophilus* strains on clear medium, as an aid for their detection in mixed cultures.

Recovery of *Haemophilus* species from 100 oropharyngeal specimens on the three different media is shown in Table 1. Selective medium A was about equal to hemoglobin-agar in detecting *H. influenzae* strains from mixed cultures. Hemoglobin-agar actually appeared to select for growth of *H. influenzae* as opposed to other *Haemophilus* species. It also appeared to exhibit a slight inhibitory action against other respiratory species. Nevertheless, *H. influenzae* was not as easy to detect on the hemoglobin-agar as on the selective medium. The clear medium did not exhibit an inhibitory tendency but appeared to permit more

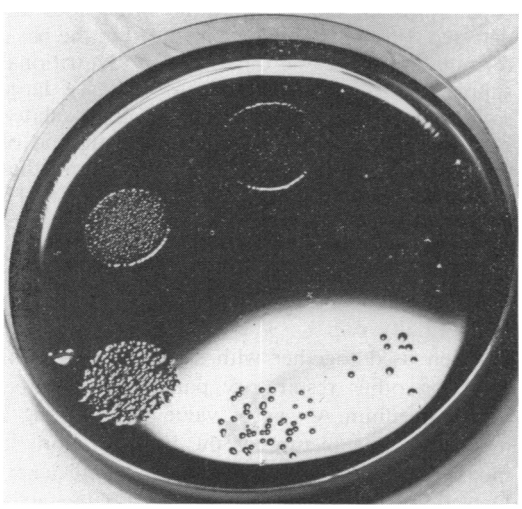

FIG. 1. *Colonies of a type b encapsulated strain of Haemophilus influenzae are shown after 24 hr of growth on Casman medium base supplemented with X and V factors and enriched with fresh rabbit blood.*

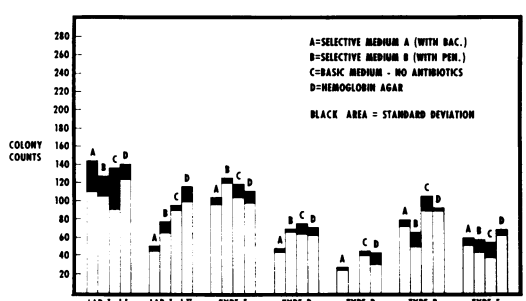

FIG. 2. *Colony counts on four test media of seven strains of Haemophilus influenzae.*

TABLE 1. *Strains of Haemophilus organisms recovered from 100 oropharyngeal swab specimens cultured on three different medium formulations*

Species	Selective medium A	Hemoglobin agar	Supplemented clear medium
H. influenzae	32	30	5[a]
H. parainfluenzae	30	9	6[a]
H. parahaemolyticus	27	3	0
H. haemolyticus	5	1	0

[a] Low incidences were due mainly to excessive overgrowth of *Haemophilus* colonies by other species on the clear medium.

growth of other oral species than was observed on routine blood-agar plate cultures used to detect pneumococci. Contaminating colonies of oral and respiratory species were limited to 10 to 20 colonies per plate on the selective medium and exceeded 200 to 300 on plates on the nonselective media.

The low-recovery results on the clear medium did not indicate lack of growth of *Haemophilus* bacteria but indicated an extensive overgrowth of *Haemophilus* colonies by other oral species, which made rapid recognition and pure culture isolation of *Haemophilus* species difficult or impossible. Smooth colony forms were cultivated, but these were rare and their iridescence did not measurably improve their separation from other colonies. The clear medium, like Levinthal medium, was effective in growing smooth colony strains of *Haemophilus* organisms in pure culture. Hemolytic strains could not be differentiated as independent from nonhemolytic strains, except on the selective medium containing fresh rabbit blood.

The *Haemophilus* colonies detected were predominately of the rough pinpoint varieties, typical of those common to upper respiratory passages. Serological typing could not be carried out by capsular swelling, but approximately 50% of the strains could be typed by agglutination by using a microtest to be described in another report.

DISCUSSION

In contrast to the oropharyngeal specimens employed in this study, most clinical specimens, such as spinal fluids, pus, etc., in which *Haemophilus* species are of greatest concern to clinicians, contain fewer contaminating oral bacteria to obscure growth of *Haemophilus* colonies on nonselective media such as hemoglobin-agar or Levinthal agar. However, on nonselective media, *Haemophilus* colonies become very difficult to detect in specimens containing a heavily mixed flora. The satellite phenomenon used in some studies to aid detection of *H. influenzae* in surveys of nasopharyngeal flora (11) does not offer the advantages of a selective medium for detection of *Haemophilus* species or permit colony counts.

Penicillin was the first antibiotic incorporated into a medium for the selective isolation of *Haemophilus* species (5). In the present study, penicillin produced erratic colony sizes. This effect was not observed with media containing bacitracin. Turk (11) used a high dilution of crystal violet in a horse blood-agar medium supplemented with commercial X and V factors as an aid to carry out an epidemiological survey of type b *H. influenzae*.

Inhibition of the selected organism as well as other bacteria on a selective medium is not uncommon. In 1948, four strains of type b *H. influenzae* were reported to be sensitive to bacitracin (4). In the present study, two of the seven *Haemophilus* strains exhibited some sensitivity to the level of bacitracin used in medium A. Other workers have found respiratory strains resistant to two units of bacitracin contained in discs that they placed on plates of routine isolation media to aid the detection of *Haemophilus* colonies in mixed cultures (3). Inhibition of *Haemophilus* growth by medium A was negligible in comparison to the relative ease with which *Haemophilus* colonies could be detected on the selective medium.

Hemolytic *Haemophilus* colonies cannot be distinguished from nonhemolytic *H. influenzae* on hemoglobin-agar or on clear media. When they occur together, one or the other often goes undetected. Fresh rabbit blood included in the basic formulation overcame this difficulty. Nutritional supplementation also helped to preserve large smooth colony forms typical of encapsulated strains. From evidence obtained with the aid of this culture medium (*unpublished data*) and that presented by Sell et al. (10) and by Austrian (1), reason exists for greater concern about the involvement of *Haemophilus* species in respiratory illness and development of methods for their control among individuals or groups with high susceptibility.

When used together with standard media for detecting other respiratory pathogens, selective culture medium A was of value in obtaining a more complete picture of the bacterial flora in mixed specimens with greater ease and efficiency. Detection of *H. influenzae* was markedly facilitated; many strains of *H. parainfluenzae* and of hemolytic species would have otherwise gone undetected. These observations suggest that such a medium could be useful for estimating the sizes of

hemolytic and nonhemolytic *Haemophilus* populations in the respiratory passages in relation to various kinds of respiratory illness, and for their qualitative detection in epidemiological investigations. The medium could be useful in evaluating the effectiveness of present and future measures to control carrier and infection rates of *Haemophilus* strains in specific populations with a high risk of infection.

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